Innate and Adaptive Gene Single Nucleotide Polymorphisms Associated With Susceptibility of Severe Inflammatory Complications in *Acanthamoeba* Keratitis

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Purpose. Over a third of patients with *Acanthamoeba* keratitis (AK) experience severe inflammatory complications (SICs). This study aimed to determine if some contact lens (CL) wearers with AK were predisposed to SICs due to variations in key immune genes.

Methods. CL wearers with AK who attended Moorfields Eye Hospital were recruited prospectively between April 2013 and October 2014. SICs were defined as scleritis and/or stromal ring infiltrate. Genomic DNA was processed with an Illumina Low Input Custom Amplicon assay of 58 single nucleotide polymorphism (SNP) targets across 18 genes and tested for association in PLINK.

Results. Genomic DNA was obtained and analyzed for 105 cases of AK, 40 (38%) of whom experienced SICs. SNPs in the *CXCL8* gene encoding IL-8 was significantly associated with protection from SICs (chr4: rs1126647, odds ratio [OR] = 0.3, $P = 0.005$, rs2227543, OR = 0.4, $P = 0.007$, and rs2227307, OR = 0.4, $P = 0.02$) after adjusting for age, sex, steroids prediagnosis, and herpes simplex keratitis (HSK) misdiagnosis. Two *TLR-4* SNPs were associated with increased risk of SICs (chr9: rs4986791 and rs4986790, both OR = 6.9, $P = 0.01$). Th-17 associated SNPs (chr1: *IL-23R* rs11209026, chr2: *IL-1β* rs16944, and chr12: *IL-22* rs1179251) were also associated with SICs.

Conclusions. The current study identifies biologically relevant genetic variants in patients with AK with SICs; IL-8 is associated with a strong neutrophil response in the cornea in AK, TLR-4 is important in early AK disease, and Th-17 genes are associated with adaptive immune responses to AK in animal models. Genetic screening of patients with AK to predict severity is viable and this would be expected to assist disease management.

Keywords: *Acanthamoeba* keratitis (AK), adaptive, complications, genetic susceptibility, inflammation, innate, keratitis, scleritis

Acanthamoeba keratitis (AK) results in the most prolonged and severe morbidity of any of the causes of acute microbial keratitis.[1] Unlike other *Acanthamoeba* infections, which usually affect immunocompromised patients, AK occurs in immunocompetent healthy individuals.[2] Treatment failure rates are currently in the order of 50% for a poor outcome (acuity less ≤ 20/80 ± surgery) with median overall cure times in the order of 5 months[3,4] and 25% of patients requiring more than 9 months of treatment.[3] Over 25% of patients in large series have required a keratoplasty.[5,6]

One of the major causes of morbidity in AK is the severe inflammatory response that develops in some patients.[7] This occurs in both the infected cornea, with the development of a corneal ring infiltrate, as well as in the usually noninfected adjacent sclera. Scleritis causes both severe pain, and, in some cases, the secondary complications of scleral thinning, choroidal detachment, and hypotony.[8–11] Corneal ring infiltrates are reported in 13 to 24% of cases and scleritis in 9 to 18% of patients with AK.[8,12,13] These severe inflammatory complications are associated with poor outcomes.[7]

We and others have shown that single nucleotide polymorphisms (SNPs) of inflammatory genes involved in animal models of bacterial keratitis are associated with the onset and severity of corneal infection in patients who wear contact lenses (CLs).[14–18] CL wear is the major risk factor for AK in developed countries, accounting for around 90% of cases.[19] This study was designed to test our hypothesis that differences in immune gene variation in otherwise healthy CL wearers determine whether a severe inflammatory response develops in the course of AK.

Table 1. Distribution of Sample, Presenting History, and Clinical Features of Patients With and Without Severe Inflammatory Complications

Characteristics	SICs $N = 40$	Non-SICs $N = 65$	*P* Value
Age at diagnosis, mean y ± SD ($n = 89$)	38.3 ± 17.3	37.9 ± 16.2	0.906
Sex, n (%) ($n = 105$)			
M	20 (50.0)	30 (46.2)	0.841
F	20 (50.0)	35 (53.8)	
Topical corticosteroid use prior to AAT *n* (%) ($n = 95$)			
No	23 (67.6)	52 (85.2)	0.065
Yes	11 (32.4)	9 (14.8)	
HSV keratitis treatment prior to AAT ($n = 95$)			
No	20 (58.8)	48 (78.7)	0.057
Yes	14 (41.2)	13 (21.3)	
Bacterial keratitis treatment prior to AAT *n* (%) ($n = 95$)			
No	13 (38.2)	25 (41.0)	0.830
Yes	21 (61.8)	36 (59.0)	
Patients referred to tertiary care (%) ($n = 103$)			
No	19 (47.5)	37 (58.7)	0.313
Yes	21 (52.5)	26 (41.3)	
Disease stage at presentation[a] (%) ($n = 84$)			
1	5 (18.5)	25 (43.9)	<0.001
2	9 (33.3)	31 (54.4)	
3	13 (48.1)	1 (1.8)	

AAT = anti-amoebic therapy; HSV = herpes simplex virus.
[a] Stage 1 corneal epitheliopathy; stage 2, stage 1 + epithelial defect, perineural infiltrate or stromal infiltrate; and stage 3, stage 2 + corneal ring infiltrate.

Methods

This was a prospective study of patients with CL associated AK classified as either displaying a severe inflammatory complication (SICs) or not (non-SICs).

Ethics

The protocol was approved by the London-Hampstead National Research Ethics Service (Reference 13/LO/0032) and Moorfields Eye Hospital Research Management Committee. The trial was adopted by the UK National Institute for Health Research, Clinical Research Network Portfolio. All participants gave written informed consent and all study procedures conformed to the tenets of the Declaration of Helsinki.

Recruitment

CL wearers who attended Moorfields Eye Hospital for diagnosis and/or ongoing treatment for AK were recruited prospectively between April 2013 and October 2014.

For the diagnosis of AK, one or more of the following criteria was satisfied:

1. A positive *Acanthamoeba* culture or histopathologic confirmation of trophozoites or cysts.
2. Culture-negative cases that were positively identified as having *Acanthamoeba* cysts on confocal microscopy, together with a typical clinical course and response to anti-amoebic treatment (AAT).
3. In the absence of criterion 1 or 2, patients with perineural corneal infiltrates or a typical clinical course with a response to AAT.

Genomic DNA was collected by a self-administered cheek swab (FLOQswabs; Copan Diagnostics, CA, USA) or a saliva kit (DNAGenotek, Ontario, Canada or DNAgard, Biomatricia, CA, USA). Age, sex, and ethnicity was collected via self-report. Clinical data were collected retrospectively from the patients' with AK hospital records using the REDCap database platform (Vanderbilt University, Nashville, TN, USA).

Disease stage at presentation of AK was divided into 3 categories: stage 1, corneal epitheliopathy only; stage 2, presence of one or more of corneal epithelial defects, perineural infiltrates or stromal infiltrates, in addition to stage 1 findings; and stage 3, presence of a corneal stromal ring infiltrate in addition to one or more features of stage 2 disease.[20]

Severe Inflammatory Complications

SICs were defined as documentation of scleritis and/or stromal ring infiltrate in the patients' hospital records in accordance with a previous study.[7]

SNP Assay Design

Much of the understanding of the immune response to *Acanthamoeba* has come from studies in animal models. These studies indicate that innate cells and proteins, such as TLR-4, IL-8, and neutrophils[21,22] are crucial to mount a response in early AK disease, and that Th17 cells are involved in limiting disease severity.[23] We therefore designed a SNP assay targeting Th-17 pathway genes (*IL17-A*, *IL17-F*, *IL17-R*, *IL-1β*, *TNFα*, *TGFβ*, *IFNγ*, *IL-6*, *IL-23R*, *IL-22*, and *IL-27*) and *CXCL8* (encodes for IL-8*)*. We also included Th-1 and Th-2 interleukins genes found associated with bacterial keratitis (*IL-12* and *IL-10*, respectively) and a novel cytokine (*IL-18*). Surface defense proteins defensin 1 and TLR-4 were further targeted, as expression of these proteins are upregulated in the presence of *Acanthamoeba* in both animal models and in vitro.[24] As a SNP of TNF-related apoptosis-inducing ligand receptor (TRAIL-R1), a negative modulator of

Table 2. Allelic Frequencies of Targeted SNPs and Association Analysis in Patients With AK With Severe Inflammatory Complications (SICs) Compared to Those Without

Gene/Protein	SNP Reference	Chromosome: Base Pair (hg38 Assembly)	Alleles A2/A1	MAF (A1)		SICs vs. Non SICs Adjusted[a]	
				SIC	Non-SIC	OR	*P* Value
IL10/IL-10	rs1800872	1:206773062	G/T	0.175	0.285	0.595	0.192
	rs1800871	1:206773289	G/A	0.175	0.292	0.569	0.160
	rs1800896	1:206773552	T/C	0.375	0.385	0.909	0.784
	rs1800890	1:206776020	A/T	0.288	0.300	0.836	0.643
IL23R/IL-23R	rs1884444	1:67168129	G/T	0.075	0.077	0.983	0.970
	rs7517847	1:67215986	T/G	0.450	0.346	2.035	0.073
	rs2201841	1:67228519	A/G	0.263	0.323	0.884	0.742
	rs11209026	**1:67240275**	**G/A**	**0.113**	**0.031**	***6.294***	***0.015***
	rs11465817	1:67255414	C/A	0.238	0.308	0.858	0.681
	rs10889677	1:67259437	C/A	0.288	0.323	0.884	0.742
IL1B/IL-1β	rs1143634	2:112832813	G/A	0.250	0.208	1.045	0.923
	rs16944	**2:112837290**	**G/A**	**0.275**	**0.362**	***0.438***	***0.031***
CXCL8/IL-8	rs4073	4:73740307	T/A	0.413	0.508	0.508	0.0703
	rs2227307	**4:73740952**	**T/G**	**0.350**	**0.500**	***0.411***	***0.019***
	rs2227549	4:73741020	A/G	0.038	0.038	1.160	0.834
	rs2227306	4:73741338	C/T	0.288	0.439	0.495	0.053
	rs2227543	**4:73742193**	**C/T**	**0.263**	**0.469**	***0.365***	***0.007***
	rs1126647	**4:73743328**	**A/T**	**0.250**	**0.462**	***0.336***	***0.005***
IL12B/IL-12 *B*	rs3212227	5:159315942	T/G	0.125	0.231	0.554	0.200
	rs10045431	5:159387525	C/A	0.300	0.339	0.775	0.466
	rs6887695	5:159395637	G/C	0.338	0.331	0.947	0.874
TNFα/TNFα	rs1800629	6:31575254	G/A	0.188	0.123	1.497	0.334
IL17A/IL-17A	rs2275913	6:52186235	G/A	0.300	0.377	0.842	0.588
	rs3748067	6:52190541	C/T	0.088	0.085	0.982	0.975
IL17F/IL-17F	rs763780	6:52236941	T/C	0.050	0.031	2.330	0.279
	rs2397084	6:52237046	T/C	0.100	0.100	0.944	0.926
IL6/IL-6	rs1800797	7:22726602	G/A	0.313	0.369	0.750	0.415
	rs1800796	7:22726627	G/C	0.113	0.108	1.074	0.886
	rs1800795	7:22727026	G/C	0.313	0.369	0.846	0.629
DEFB1/DEFβ1	rs1800972	8:6877901	G/C	0.175	0.246	0.701	0.395
	rs1799946	8:6877909	C/T	0.375	0.346	1.349	0.437
	rs2702877	8:6878545	C/G	0.275	0.269	1.020	0.957
	rs5743409	8:6879098	C/A	0.425	0.423	0.937	0.841
TRAIL-R1/TNFRSF10A	rs20576	8:23200707	T/G	0.175	0.185	1.253	0.605
	rs20575	8:23201811	G/C	0.375	0.485	0.739	0.346
	rs6557634	8:23202743	C/T	0.363	0.477	0.711	0.284
TLR4/TL-R4	rs10983755	9:117702392	A/G	0.038	0.015	4.065	0.282
	rs10759932	9:117702866	T/C	0.163	0.162	0.785	0.630
	rs11536879	9:117709933	A/G	0.038	0.054	0.808	0.787
	rs1927907	9:117710486	C/T	0.150	0.169	0.814	0.674
	rs4986790	**9:117713024**	**A/G**	**0.100**	**0.031**	***6.915***	***0.014***
	rs4986791	**9:117713324**	**C/T**	**0.113**	**0.031**	***6.915***	***0.014***
	rs11536889	9:117715853	G/C	0.163	0.162	0.780	0.593
	rs7873784	9:117716658	G/C	0.175	0.169	1.412	0.403
IL18/IL-18	rs187238	11:112164265	C/G	0.250	0.269	0.761	0.512
IFNγ/IFNγ	rs2069718	12:68156382	G/A	0.425	0.369	1.302	0.445
	rs1861494	12:68157629	T/C	0.300	0.300	0.935	0.826
	rs2430561	12:68158742	T/A	0.463	0.462	1.109	0.755
IL22/IL-22	**rs1179251**	**12:68251271**	**C/G**	**0.025**	**0.131**	***0.0620***	***0.014***
	rs2227485	12:68253933	A/G	0.475	0.408	1.216	0.567
	rs2227478	12:68254842	A/G	0.400	0.292	1.996	0.0665
	rs2227473	12:68255258	C/T	0.113	0.131	1.243	0.666
	rs2227472	12:68255353	C/T	0.463	0.415	1.174	0.634
IL27/IL-27	rs4788084	16:28528527	C/T	0.375	0.323	1.294	0.423
TGFB1/TGFβ	rs1800471	19:41352971	C/G	0.063	0.085	0.530	0.353
	rs1800469	19:41354391	G/A	0.313	0.277	1.178	0.666
IL17RA/IL-17RA	rs4819554	22:17084145	A/G	0.238	0.192	1.428	0.387
	rs2229151	22:17108407	G/A	0.025	0.023	1.288	0.758

Statistically significant differences in bold typeface.

MAF = minor allele frequency.

[a] SICS versus age at diagnosis, sex, topical corticosteroid use prior to anti-amoebic therapy and HSV keratitis treatment prior to anti-amoebic therapy.

Table 3. Genotype Frequencies of *CXCL8* (Encodes IL-8) and *TLR-4* in Patients With Severe Inflammatory Complications (SICs) and Controls

Gene/Protein	SNPs	Genotypes	SICs (%) ($n = 40$)	Non-SICs (%) ($n = 65$)	Adjusted *P* Value (Dominant Model)
CXCL8/IL8	rs2227543 C/T4:73742193	TT	3 (7.5)	14 (21.5)	0.0145
		TC	15 (37.5)	33 (50.8)	
		CC	22 (55.0)	18 (27.7)	
	rs1126647 A/T4:73743328	TT	2 (5.0)	14 (21.5)	0.0111
		TA	16 (40.0)	32 (49.2)	
		AA	22 (55.0)	19 (29.2)	
TLR4/TLR4	rs4986790 A/G9:117713024	GG	0	0	0.0541
		GA	8 (20.0)	4 (6.2)	
		AA	32 (80.0)	61 (93.8)	
	rs4986791 C/T9:117713324	TT	0	0	0.0290
		TC	9 (22.5)	4 (6.2)	
		CC	31 (77.5)	61 (93.8)	

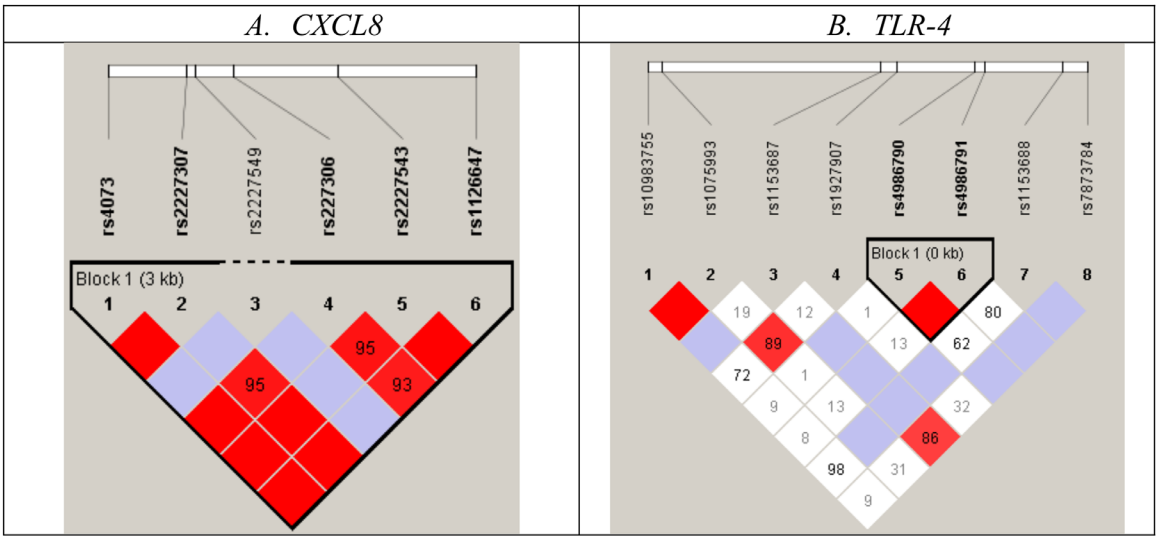

Figure. Linkage disequilibrium structure of selected SNPs in regions of interest in *CXCL8* (encodes IL-8) (**A**) and *TLR-4* (**B**). LD blocks were created with the default algorithm in HaploView program (version 4.1) that creates 95% confidence bounds on the normalized coefficient of linkage disequilibrium (D′) considered being in strong LD where 95% of the comparisons made are informative. The coloring of the boxes depends on two scores, the logarithm of the odds (LOD) and D′, high association (LOD > = 2 and D′ = 1, *red*), low association (LOD < 2 and D′ = 1, *blue*), and no association (LOD < 2 and D′ < 1, *white*). LOD score are shown within the box if D′ < 1.

inflammation, has recently been associated with trachoma infection in humans[25] and it was also included in the SNP panel.

Following collection via a cheek swab or a saliva kit, genomic DNA was extracted and processed using the Illumina MiSeq platform. Detailed gDNA sample processing and bioinformatics methods are included in Supplementary File S1.

SNPs and Phenotype Association Analysis

The PLINK (version 1.07) software was used for association analysis.[26] SNPs with a minor allele frequency < 0.01 in the study cohort or not conforming to Hardy-Weinberg Equilibrium ($P < 0.001$) were removed. Remaining SNPs were assessed for association with SICs using logistic regression. Topical corticosteroid use prior to the initiation of AAT, bacterial keratitis treatment prior to AAT, herpes simplex virus (HSV) keratitis treatment prior to AAT, disease stage at presentation, and tertiary referral (i.e. referred from another hospital for tertiary management at Moorfields Eye Hospital) were tested for univariate associations and included in the multivariate model if significant ($P < 0.2$) along with sex and age as covariates. Analysis of linkage disequilibrium between multiple SNPs in the same gene was performed using HaploView (version 4).[27]

Results

Genomic DNA was obtained and analyzed for 105 cases of AK, 40 (38%) of whom experienced SICs (Table 1). The average age at the time of presentation for all AK cases was 36.9 ± 15.5 years, and there was no difference in age between those with SICs compared to those without (38.3 ± 17.3 compared to 37.9 ± 16.2, $P = 0.906$). Likewise, there was no difference in sex between the two groups (women: 20/40, 50% vs. 35/65, 54%, $P = 0.841$). "Topical corticosteroid use" and "herpes simplex virus (HSV) keratitis treatment prior to the initiation AAT" was significant $P < 0.2$ and was included in the genotype model. "Disease stage at presentation" was more advanced in the SICs group ($P < 0.001$) so this variable was not included

in the final model due to collinearity. There was no difference between "bacterial keratitis treatment prior to AAT" and "patients referred to tertiary care" for SICs versus non-SICs and so these characteristics were also not included in the multivariate model.

Of the 72 targeted SNPs, 6 were not able to be sequenced with the assay (*IL1B* rs1143627; *IL10* rs6703630; *TLR4* rs12377632; *IL18* rs5744292; *IL22* rs2227483; and *IFNγ* rs2069707), 5 did not conform to Hardy-Weinberg Equilibrium ($P > 0.001$, *IL12B* rs17860508; *TNFα* rs1799964; *IL22* rs1012356; *IFNγ* rs2069705; and *TGFB1* rs1800470), and three had minor allele frequencies of < 0.01 (*CXCL8*/IL-8 rs2227532; *TRAIL-R1* rs2230229; and *IFNγ* rs2069709) in this cohort. The remaining 58 SNPs were included in the analysis. Table 2 details the allele frequencies of the analyzed SNPs and adjusted multivariable analysis by allele. The adjusted analysis accounted for the a priori factors of age at diagnosis, sex, topical corticosteroid use prior to AAT, and HSV keratitis treatment prior to AAT. Significant associations (adjusted $P < 0.05$) after adjustment for covariates were found in SNPs in genes *IL-23R*, *IL-1β*, *CXCL8*, *TLR-4*, and *IL-22*.

The highest significance was found for *CXCL8* (encodes IL-8) and *TLR-4* SNPs and because an association was found with more than one SNP for each of these genes, they were investigated further for inheritance and linkage disequilibrium (LD).

Associated SNPs in *CXCL8* (encodes IL-8) and *TLR-4* demonstrated dominant inheritance models (Table 3). LD was performed in HaploView (version 4) for the targeted SNPs in *CXCL8* (encodes IL-8) and *TLR-4* (Fig.). All SNPs in *CXCL8* (encodes IL-8) were in LD apart from rs2227549 (4:73741020). For *TLR-4* only the 2 associated SNPs were in an LD block with low LD across the rest of the gene.

Discussion

This study investigated SNPs in genes encoding innate and adaptive immune responses thought to play key roles in the severity of AK from in vitro and in vivo animal and human studies. SNPs in five genes encoding biologically relevant molecules, *CXCL8* (encodes IL-8, rs2227543 C/T, rs1126647 A/T, and rs2227307 T/G, all in 3'UTR gene region) and *TLR-4* (rs4986790 A/G, rs4986791 C/T, missense coding gene region), *IL-1β* (rs16944, G/A, upstream regulatory region), *IL-23R* (rs11209026, G/A, missense coding gene region), and *IL-22*(rs1179251, C/G, intron gene region) were identified that were associated with SICs in AK after adjusting for covariates. SNPs of *CXCL8* (encoding IL-8) and *TLR-4* had two significantly associated SNPs, each in LD and exhibited the strongest association with SICs.

The *CXCL8* (encodes IL-8) SNPs were protective for SICs in this population. Carriers of the minor allele (T) were around 70% less likely to experience SICs than noncarriers in this study. In this same AK patient cohort, increased levels of IL-8 protein in tears were associated with more severe AK disease.[28] However, tear IL-8 levels with were not correlated with specific genotypes in patients in this study ($P = 0.324$). Similarly, a study of sepsis susceptibility in Chinese men[29] found that the T allele for SNP rs1126647 was protective and increased levels of serum IL-8 were associated with sepsis in their population, but found no correlation between specific genotypes and the serum IL-8 protein levels. These findings indicate that this SNP may not be the causal SNP that increases the transcriptional capacity for IL-8. Other studies have suggested that rs4073 and rs2227306 may be causal SNPs.[30] Although significance associations of these SNPs were not found in this study, they are in LD with those identified as significant.

IL-8 is produced in the cornea by epithelial cells, keratocytes,[31] and macrophages[22] and has been shown to be upregulated in other ocular surface diseases, such as allergy[32] and dry eye.[33] IL-8 mobilizes and activates neutrophils, which is crucial in the early stage of corneal infection. However, activated neutrophils can also release metalloproteinases, which can degrade the extracellular matrix.[34] This is evidenced by an immunodeficient mouse model, which when infected with adenovirus vector encoding human IL-8, results in neutrophil infiltration and corneal ulceration.[35] Thinning of the cornea and persistent epithelial defects, whereas not by definition part of SICs, are often present in severe cases of AK.[19] IL-8 is produced early in the inflammatory cycle, but unlike most other inflammatory cytokines, it can remain active up to weeks at the site of inflammation.[36,37] In contrast to the transitory effect of other proinflammatory cytokines that may last only a few hours, IL-8 may have a sustained influence[36,37] and therefore could be an important contributor to severe inflammatory outcomes in AK. In addition, IL-8 may be repeatedly produced by epithelial and inflammatory cells, leading to prolonged inflammation and collateral tissue damage.

Although IL-8 may have detrimental proinflammatory effects mediated through neutrophils, several animal studies have shown the powerful anti-acanthamoeba action of neutrophils in the early stages of disease. Neutrophils are chemotactic to *Acanthamoeba* trophozoites and cyst lysates, and are capable of killing the organism in vitro through myeloperoxidase (MPO).[38] Upon injection of one million *Acanthamoeba castellani* trophozoites into the anterior chamber of a mini pig model, AK was eliminated by a robust neutrophil response.[39] Intraocular infection has only been reported in a handful of human cases[8] indicating this neutrophil response in the anterior segment may be a factor in limiting the organism from infecting the posterior eye in humans.

In contrast to *CXCL8*, minor alleles of *TLR-4* SNPs were associated with more severe AK in this study. Carriers of the minor allele were around 6.9 times more likely to experience SICs than noncarriers. The two significant *TLR-4* SNPs are in almost complete LD. These SNPs are not common, however, the non-SIC minor allele frequencies are similar to population controls (minor allele frequency 0.06 from phase III 1000 Genomes) with increased frequency in SICs, reflecting a representative sample.

TLR-4 is a surface recognition receptor present on corneal and conjunctival cells. In the presence of *Acanthamoeba*, in vivo and in vitro models both show activation of TLR-4 receptor.[40] In mouse models of AK, TLR-4 is also involved in initiating the cytokine complex.[40] It is likely that in patients with AK, decreased recognition of *Acanthamoeba* allows a greater inoculum load into the cornea with dysregulation of the cytokine response, increasing disease severity. TLR-4 is primarily associated with a pro-inflammatory response, however, chronic activation of TLR-4 can lead to an anti-inflammatory response in some circumstances.[41] If this is the case in AK, patients with milder disease may be able to mount this anti-inflammatory response, whereas those with the SNP and more severe disease may not, leading to chronic hyperinflammation.

A study of patients with bacterial keratitis in India found higher carriage of *TLR-4* rs4986791 SNP compared with healthy controls.[18] However, another study did not find an association in rs4986790 (in LD with rs4986791) between CL patients with bacterial keratitis compared with healthy CL wearing controls in a Caucasian population.[14] The differences in results in these two studies are intriguing, but may be attributable to the rarity of the minor alleles. Interestingly, carriers of *TLR-4* SNPs have been shown to have a decreased response to endotoxins in several studies.[42,43] Of note, patients with *TLR-4* SNPs show a blunted airway response to inhalation of lipopolysaccharide (LPS).[42] The minor (T) allele of rs4986791 is associated with lower TLR-4 expression in blood plasma of a large German cohort, which is replicated in a Arab and Asian cohort of healthy individuals.[44]

Three Th17-related genes were associated with SICs in this study, *IL-1β*, *IL-23R*, and *IL-22*. IL-1β and IL-22 are proinflammatory cytokines and associated with the differentiation of $CD4^+$ T cells into effector Th17 cells. *For IL-22*, the SNP (rs1179251), as for *CXCL8* (encodes IL-8) was protective. IL-22 tear fluid protein was also expressed more often in this cohort of patients with AK who experienced severe disease, as for IL-8.[28] However, we did not find an association between IL-22 tear protein levels and rs1179251 genotypes ($P = 0.07$). To our knowledge, there are no studies that correlate IL-22 protein to rs1179251. Of note is that serum IL-22 protein is increased in idiopathic scleritis.[45] A study of IL-22 gene variations in autoimmune patients found the CC wild type genotype was associated with Graves' ophthalmopathy, conferring protection by the minor allele, similar to our findings.[46] Serum levels of IL-22 are associated with the minor allele of rs2227484,[47] and stimulated peripheral blood mononuclear cells (PMBC) from patients carrying the minor allele of rs2227473 produce more IL-22.[48] However, both these SNPs are in the promotor region of the gene, whereas rs1179251 is in intron 4, rendering it difficult to draw comparisons.

In alignment with other cytokines in this study, the significant detection of SNP in *IL-1β* (rs16944) is associated with a lower risk of SICs. This SNP minor allele is associated with increased gene expression in PBMCs.[49] However. IL-1β tear protein was not detectable in any of the tear samples from AK cases or nonaffected healthy controls in this current study population.[28] This SNP and others in *IL-1β* have been associated with susceptibility to early onset periodontitis[50] and osteoarthritis.[51] In a previous study in a Caucasian population, an association was not found with *IL-1β* SNPs between patients with CL associated bacterial keratitis and healthy CL wearing controls.[14] Further work is required to understand the role of *IL-1β* in AK.

For *IL-23R* rs11209026, carriage of the A allele was associated with a 6 times increased risk of SICs. The presence of the A allele increases expression of the soluble form of IL-23R mRNA (which then functions as a decoy receptor) and thus impairs the function of IL-23 in its ability to promote and maintain Th-17 cells.[52] This may mean that the Th-17 responses observed in an animal model of AK[23] are hampered in patients with this SNP.

There are several limitations of this study, in particular the sample size. Although this is a rare disease and we have a relatively large sample size per se, this limits power. In addition, the small sample size did not enable a more comprehensive analysis, such as genomewide association study (GWAS) or exome screening. Similarly, we have not made a correction for multiple testing of the 58 SNPs. All of the SNPs analyzed in this study had a priori hypothesis of association based on previous literature, but none reach significance under strict multiple testing criteria, despite the large effect sizes in many instances. Replication of these findings in similar, but independent, cohorts is critical and meta-analyses will likely be required to reach true statistical significance. Another limitation in this rare cohort was the self-report of ethnicity, which was completed poorly by participants (76/105, 72% identified ethnicity), however, 70/76, 92% identified as Caucasian. In a previous genetic study of bacterial keratitis in CL wearers at Moorfields Eye Hospital in 2012, 82% were Caucasian,[14] adding certainty that the cohort in this current study is predominantly Caucasian.

Nevertheless, the study author's clinical experience in this area meant a very well phenotyped cohort. In addition, a well-matched cohort in terms of sex and age between the SICs and non-SIC groups were recruited. Our results are further strengthened by an earlier published tear fluid protein study carried out on the same patient cohort.[28]

In summary, our study has highlighted the importance of innate and Th17 immunity in patients with AK. A study of gene expression in late stage bacterial and fungal keratitis also found persistence of innate immune pathways.[53] Further work will be important to define cause and effect in tissue and animal models.

Furthermore, this study demonstrates that genetic screening of patients with AK for the presence of these SNPs is viable and could predict the risk of patients developing the severe inflammatory complications of the disease. The presence of these SNPs in an individual patient could assist clinicians managing AK by identifying patients susceptible to developing SICs in whom the prompt treatment of inflammation can be expected to improve outcomes.

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